



## OXYGEN-HEMOCYANIN RELATIONSHIPS IN THE LAND CRAB, *CARDISOMA GUANHUMI*<sup>1</sup>

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Relatively little quantitative information is available concerning the *in vivo* oxygen levels in the blood of the decapod Crustacea. Investigations upon three decapods, the spiny lobster, *Panulirus interruptus*, the American lobster, *Homarus americanus*, and the sheep crab, *Loxorhynchus grandis*, indicated that the total oxygen content of the blood was low and that the oxygen tension in both pre-branchial and post-branchial blood samples was not over 12 mm Hg. These low oxygen tensions resulted from the failure of the hemocyanin in the above forms to become saturated with oxygen as the blood passed through the gills. The hemocyanin was found to be responsible for most of the oxygen transported to the tissues since the blood, operating at very low oxygen pressures, could carry very little in physical solution (Redmond, 1955). Zuckerkandl (1957a, 1957b), on the other hand, found in the crab, *Maia squinado* (= *Maja*), that during certain stages of the molt cycle the hemocyanin might disappear completely, and at this and other times the oxygen tension in the post-branchial blood was considerably higher than in the previously mentioned three species. The present study of the land crab, *Cardisoma guanhumi* Latreille, was undertaken to add to the knowledge of blood-oxygen relationships in living decapods and to extend these observations to an organism from an environment unlike that of the above species.

### OXYGEN EQUILIBRIUM CURVES

Specimens of *Cardisoma* were collected from two locations, the Port Royal area of Jamaica, W. I., and Coral Gables, Florida. Blood samples to be used in the determination of oxygen equilibrium curves were withdrawn from the bases of the walking legs by means of 5-ml. hypodermic syringes. The blood was allowed to clot and was then expressed through cheese-cloth. Only freshly drawn blood was used. When necessary, the pH of the blood was adjusted with borate or Tris buffers; otherwise, the blood was untreated. Standard borate solutions were mixed one-to-one with the blood samples and were used in a first series of measurements. In later measurements two concentrated solutions of Tris in sea water (1 gram Tris:10 ml. water), adjusted with HCl to pH 8.5 and pH 6.7, were added to the blood in small quantities to achieve various levels of pH without appreciably changing the concentration of the hemocyanin. The pH of the blood samples was taken after the blood had been equilibrated with air, immediately prior to the

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determination of the oxygen equilibrium curves. Much of the diffusible carbon dioxide would have been lost at this time; however, the possibility exists that additional carbon dioxide loss at low pressures may have resulted in a final pH somewhat higher than that recorded originally. Comparison of the oxygen equilibrium curves of undiluted blood with blood of the same pH to which these buffers had been added, indicated that, at least at normal blood pH, the buffers did not noticeably alter the curve.

Oxygen equilibrium curves were determined by the vacuum pump-spectrophotometric method described previously (Redmond, 1955), or by a modification of this procedure similar to that of Jones (1954). In the original procedure a cylindrical cuvette containing the blood was connected by pressure tubing to a vacuum pump. This connection was maintained throughout the determination, and equilibration was achieved by gently shaking the cuvette by hand. In the modified procedure the cuvette is replaced by one or more small tonometers which can be sealed and disconnected from the pressure tubing after the desired internal pressure has been reached. One end of the tonometer consists of an exchangeable cuvette which can be placed directly in the well of a Coleman Jr. spectrophotometer. Equilibration is effected by placing the tonometer in a slowly moving (62 cycles/min.) reciprocal shaker. The latter method has several advantages. In addition to not requiring the constant attention of the investigator during equilibration, more than one curve may be determined simultaneously. Longer periods of equilibration are required, but, since the shaking is slower and more controlled, less cloudiness due to protein denaturation occurs. Another important factor is that, because the tonometer is sealed, it is easier to maintain consistent low pressures. Depending upon the size of the cuvette portion of the tonometer, 0.5 to 3.0 ml. of blood were required for each curve. In the present series of experiments, optical density readings were made at 575 millimicra, the wave-length of maximum absorption for the oxyhemocyanin of *Cardisoma*. Temperatures were maintained at  $\pm 0.5^\circ$  C. At pH 7.5 and  $25^\circ$  C. the hemocyanin of *Cardisoma* becomes half-saturated with oxygen at a partial pressure of about 3.5–4 mm. Hg (Figs. 1 and 2). This is an unusually high oxygen affinity for a crustacean hemocyanin. Only one other crustacean hemocyanin, that of the crayfish, *Procambarus simulans*, has been reported to have as low a half-saturation pressure. Latimer (1961) found this hemocyanin to become half-saturated at 3.5 mm.  $O_2$  at  $25^\circ$  C. and pH 7.7. The possible significance of this high affinity will be discussed later.

Figure 1 illustrates representative oxygen equilibrium curves and Figure 2 indicates how the pressure of half saturation varies with changing pH. Both figures show that this pigment, like all other known crustacean hemocyanins, possesses a normal Bohr effect. For purposes of comparison the extent of this shift in half-saturation with pH can be expressed by the equation

$$\phi = \Delta \log p_{50} / \Delta \text{pH} \quad (\text{Wyman, 1948}).$$

For the interval pH 7.3 to pH 7.8, at  $25^\circ$  C., *Cardisoma* hemocyanin has a value of  $\phi = -0.75$ . Since there are so few comparable measurements it is difficult to judge whether or not this is a normal figure for decapod Crustacea. For the spiny lobster, *Panulirus interruptus*,  $\phi = 0.30$  (Redmond, 1955). What little evidence exists suggests that the values of  $\phi$  for crustacean hemocyanins probably lie in the

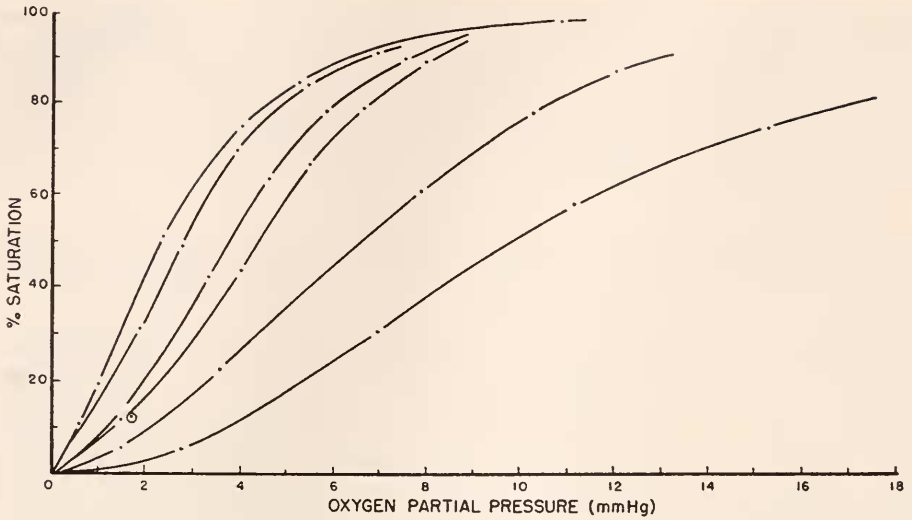


FIGURE 1. Oxygen equilibrium curves for the hemocyanin of *Cardisoma guanhumi* at 25° C. The pH at which each curve was determined is, from left to right, 7.90, 7.70, 7.54, 7.41, 7.30, 7.01.

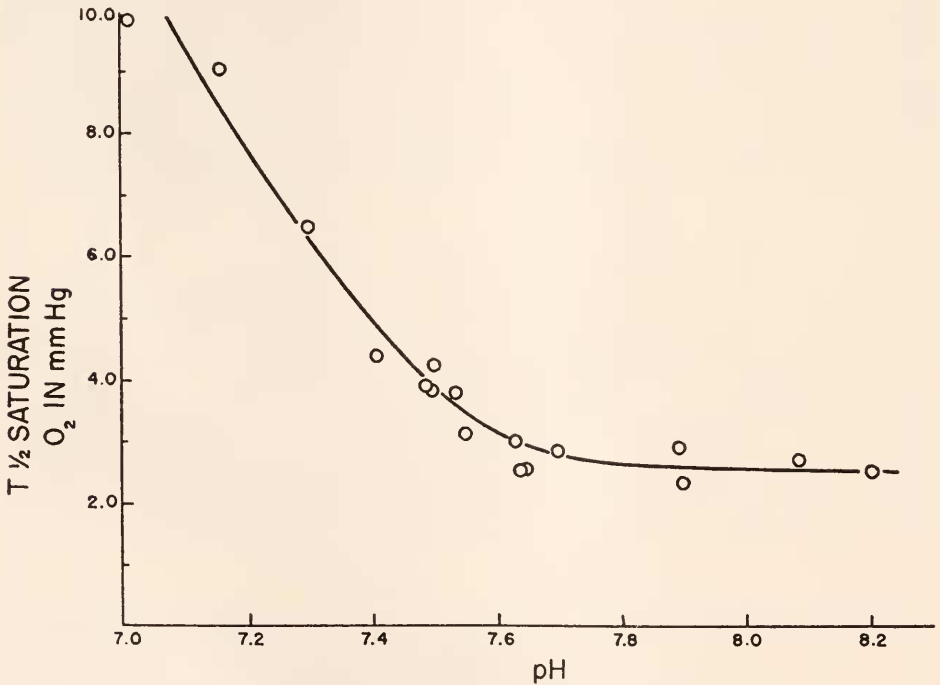


FIGURE 2. The effect of pH upon the pressure at which the hemocyanin of *Cardisoma guanhumi* becomes half-saturated. 25° C.

TABLE I

Values of "n" for oxygen equilibrium curves of the hemocyanin of *Cardisoma guanhumi* at normal pH

pH	"n"
7.41	2.81
7.50	2.69
7.54	2.50
7.55	2.73
7.63	2.31
7.64	2.78
Av. 7.55	2.64

range of those found for vertebrate hemoglobins; horse  $-0.60$  (St. George and Pauling, 1951), surf duck  $-0.58$ , western grebe  $-0.45$  (Manwell, 1958), mackerel, *Scomber scomberus*,  $-1.02$  (Nicol, 1960). The latter values are calculated for the pH range 7.0–7.5. The crustacean hemocyanins would have appreciably higher values over this range, but since the pH of their blood is usually near 7.5, the range 7.3–7.8 would appear to be of more physiological interest.

The sigmoid shape of the oxygen equilibrium curves of *Cardisoma* hemocyanin indicates that interaction occurs among the oxygen-combining sites of the hemocyanin molecule. Plotting the oxygen equilibria data in the form of  $\log p$  vs.  $\log (y/100 - y)$ , where  $p$  is the partial pressure of oxygen and  $y$  is the corresponding per cent saturation, yields a line whose slope, "n," is an approximation of the extent of interaction (Klotz, 1954). Six values of "n" (Table I) at normal blood pH average 2.64. Thus a considerable degree of positive facilitation occurs in the binding of oxygen by the different sites of the *Cardisoma* hemocyanin molecule.

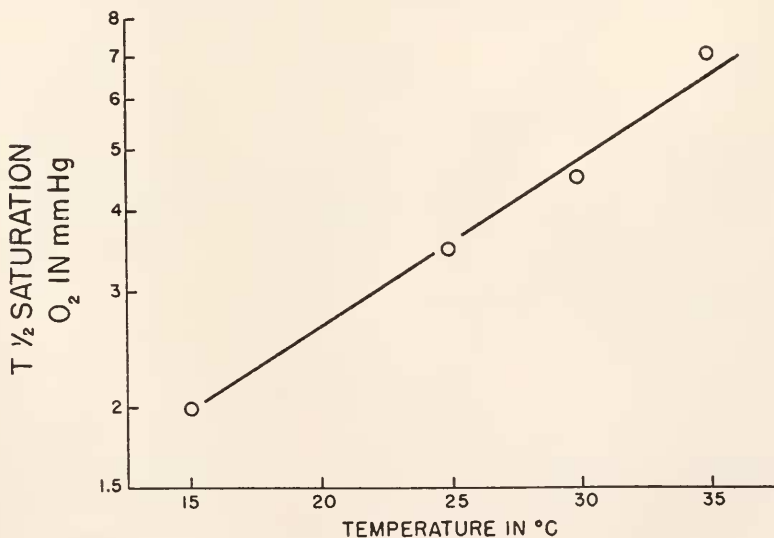


FIGURE 3. The effect of temperature upon the pressure at which the hemocyanin of *Cardisoma guanhumi* becomes half-saturated; pH 7.53.

Figure 3 illustrates the effect of changing temperature upon the pressure of half-saturation. The slope of the graph is similar to that of several other hemocyanins (unpublished data), but, because of the high affinity of *Cardisoma* hemocyanin for oxygen, the absolute change in half-saturation pressure is small. At 15° C. the pigment becomes half loaded at 2 mm. Hg of oxygen, while at 35° C. this figure has increased only to 7 mm.

## BLOOD-GAS ANALYSES

Direct analyses of oxygen and carbon dioxide in the blood were carried out by the Van Slyke constant volume manometric technique (Consolazio, Johnson and Marek, 1951). One-ml. samples of blood were used, with 5% KCN and 1 N lactic acid being used to free the oxygen and carbon dioxide, respectively (Redmond, 1955). Post-branchial blood was taken by means of a 2-ml. hypodermic syringe from the pericardium, pre-branchial blood from the ventral thoracic sinus. Table II summarizes the results of the oxygen analyses. These measurements were made at 29° C. on freshly captured Jamaican crabs, all of which were in stage C<sub>4</sub> of the molt cycle. The water of Kingston Harbor and the daytime air temperature were both 29° C. The oxygen capacity of the hemocyanin was approximated by subtracting, from the oxygen capacity of the whole blood, 90% of the amount of oxygen that would dissolve in sea water at 29° C. The per cent saturations of the hemocyanin in the pre-branchial and post-branchial blood samples were calculated

TABLE II

Oxygen levels in pre-branchial (V) and post-branchial (A) blood of *Cardisoma guanhumi*

Species	Oxygen capacity*		In vivo oxygen content								
	Whole blood Vol. % O <sub>2</sub>	Hcy Vol. % O <sub>2</sub>	Total vol. % O <sub>2</sub>			Hcy % sat.		O <sub>2</sub> pressure** mm. Hg		% of total O <sub>2</sub> as oxy hcy	
			A	V	A-V	A	V	A	V	A	V
<i>Cardisoma guanhumi</i>	3.01	2.61	1.65	0.87	0.78	62	30	5.1	3.0	99	99
	2.55	2.15	1.75	0.99	0.76	81	35	6.9	3.4	99	99
	2.93	2.53	1.57	0.85	0.72	61	33	5.1	3.2	99	99
Average	2.83	2.43	1.66	0.90	0.75	68	33	5.7	3.2	99	99
<i>Panulirus interruptus</i> ***											
Average of nine specimens	1.99	1.53	0.82	0.35	0.46	54	22	7	3	96	97
<i>Loxorhynchus grandis</i> ***											
Average of five specimens	1.03	0.58	0.41	0.17	0.24	68	30	8	3	90	94
<i>Homarus americanus</i> ***											
Average of two specimens	1.31	0.86	0.44	0.18	0.26	49	20	5	2	95	95

\* Oxygen content following equilibration with air at 29° C. at atmospheric pressure.

\*\* Since the pH values of the blood samples were not determined, the calculations of post-branchial oxygen pressures from an oxygen equilibrium curve determined at pH 7.53 renders these pressures uncertain by approximately +1.5 to -1.0 mm. Hg. For reasons detailed in Redmond (1955), the A-V pH shift is believed not to be great enough to significantly affect these calculations.

\*\*\* The data for *Panulirus*, *Loxorhynchus*, and *Homarus* are taken from Redmond (1955).

by trial addition of the oxygen equilibrium and oxygen solubility curves until the sums were equal to the amounts of oxygen indicated by the Van Slyke analyses. At 29° C. and pH 7.53 the oxygen equilibrium curve has a half-saturation value of 4.5 mm. oxygen. Using this curve and the per cent saturations the partial pressures of oxygen in the blood samples can be obtained.

From the table it can be seen that in both pre-branchial and post-branchial blood samples, as a result of the unsaturated condition of the hemocyanin and the high oxygen affinity of this pigment, oxygen pressures are very low and practically all of the oxygen present is in combination with the hemocyanin. In each of the three cases about 0.75 ml. of oxygen was delivered to the tissues by 100 ml. of blood. The oxygen capacities of the whole bloods are higher than those reported for most other decapod crustacea and, correlated with this, samples of oxygenated blood were deep blue in color.

Blood for carbon dioxide analysis was taken from the bases of the legs of 5 specimens of *Cardisoma*. The 5 analyses yielded an average of 49.4 vol.% total carbon dioxide, the individual measurements being 46.0, 54.0, 46.8, 42.6 and 57.7 volumes per cent. These appear to be the greatest *in vivo* carbon dioxide concentrations reported for a decapod crustacean and are 2-5 times as large as figures given for other members of this group (Collip, 1920; Parsons and Parsons, 1923; Redmond, 1955; Wolvekamp and Waterman, 1960). Possibly this high concentration of carbon dioxide may be a result of aerial respiration plus a large alkaline reserve. The vast bulk of this carbon dioxide is presumably present as bicarbonate. The partition between dissolved gas and bicarbonate, however, cannot be established without first determining a carbon dioxide dissociation curve. This has not yet been done. Since *Cardisoma* is primarily aerial in respiration and the partial pressures of gases in the branchial chamber are unknown, it is not possible at present to make a reasonable estimate of the carbon dioxide tension in the blood.

#### DISCUSSION

The oxygen equilibria data for the hemocyanin of *Cardisoma guanhumii* are qualitatively similar to those of the hemocyanins of other decapod crustacea. The sigmoid shape of the equilibrium curve and the presence of the normal Bohr effect are typical (Stedman and Stedman, 1925, 1926a, 1926b; Redfield, Coolidge and Hurd, 1926; Hogben and Pinhey, 1927; Redfield and Ingalls, 1933; Redfield, 1934; Redmond, 1955; etc.). Unusual, however, is the high oxygen affinity possessed by this pigment. Ordinarily, blood respiratory pigments having very low half-saturation pressures are associated with rather sluggish organisms or with ones living in areas where the environmental oxygen levels may be low (Prosser *et al.*, 1950). Neither of these situations appears to fit *Cardisoma*. Moving about on the surface of the ground, *Cardisoma* is nimble and its attitude is one of alertness. When disturbed it can move with considerable speed. Certainly, being an air breather, it need not experience low external oxygen pressures. The question then arises as to what, if any, is the significance of the high oxygen affinity of its hemocyanin.

One possibility is that it may be secondarily related to the aerial habits of this crab. One of the major problems facing *Cardisoma*, as a marine organism only

partially adapted to a terrestrial life, must be the prevention of desiccation due primarily to water loss from the respiratory surfaces. Associated with this, most species of intertidal crabs which spend time out of water show a reduction in gill surface which is correlated with the per cent of time spent in air (Gray, 1957; Waterman, 1960). While other modifications also occur, such as vascularization and outgrowths of the branchial chamber lining to form new respiratory surfaces, adaptations of the branchial chamber which reduce water loss will almost necessarily also reduce the over-all rate at which oxygen will diffuse into the blood. Since the rate of diffusion is directly proportional to the partial pressure gradient, the farther to the left the oxygen equilibrium curve, the faster will oxygen penetrate. This, of course, results from the combination of the oxygen with the hemocyanin as the oxygen enters the blood, the internal partial pressure thus being kept very low. This maintenance of a high diffusion gradient may be the most important respiratory function of the blood respiratory pigments in many invertebrates, the increased oxygen-carrying capacity in such forms being perhaps of only secondary importance. The high oxygen affinity of *Cardisoma* hemocyanin may then represent a secondary adaptation compensating for the effect of water-conserving measures on oxygen uptake.

Another possibility, since practically nothing is known about the hemocyanins of tropical species, is that the low half-saturation pressure may be related to the relatively high environmental temperatures experienced by such forms. In this connection, it is interesting to note that a high oxygen affinity acts as a temperature buffer tending to stabilize loading pressures. As was previously mentioned, when the half-saturation pressures of the hemocyanins of a number of species are plotted against temperature on a semi-log graph, the resulting lines are roughly parallel. This means that absolute change in half-saturation per degree centigrade will be least in those blood respiratory pigments having the highest oxygen affinities. In the case of the hemocyanin of *Cardisoma* the changes are so small that, for environmental temperatures, the half-saturation pressure can almost be considered temperature-independent. However, without more data on the ecology of this organism, it is difficult to determine whether or not this feature is of physiological importance. The high oxygen affinity of *Procambarus* hemocyanin may similarly be an adaptation to fluctuating environmental temperatures and possibly oxygen concentrations.

The Van Slyke oxygen analyses of pre-branchial and post-branchial blood samples represent only three animals so that the average figures given in Table III may or may not reflect the population as a whole. In principle, however, the results agree completely with those of previous studies on three other species of decapod crustaceans, *Panulirus interruptus*, *Homarus americanus*, and *Loxorhynchus grandis* (Table II). In none of these species was the hemocyanin found to become saturated with oxygen as the blood passed through the gills. Consequently, the oxygen pressures in both pre-branchial and post-branchial blood samples were very low. Because of the high oxygen affinity of *Cardisoma* hemocyanin, post-branchial oxygen pressures (5-7 mm. Hg) were lowest in this species. The pre-branchial oxygen levels were very similar in all four species, being in the neighborhood of 2 to 4 mm. Hg.

About 0.75 volume of oxygen is delivered to the tissues by 100 volumes of

*Cardisoma* blood. This is a little more than that found for the other three forms but still indicates that only a small quantity of oxygen is delivered by a relatively large volume of blood. Since the hemocyanin of *Cardisoma* does not become saturated and the half-saturation pressure is very low, practically all of the oxygen carried by the blood is in combination with the hemocyanin. In practice, then, the hemocyanin of *Cardisoma* transports nearly all of the oxygen used by the tissues. Despite this finding there is still some question as to how essential is hemocyanin for oxygen transport in the decapod crustacea.

Zuckerkindl (1957a, 1957b) reported the very interesting finding that during certain stages of the molt cycle of the spider crab, *Maia squinado*, the hemocyanin concentration of the blood may drop to essentially zero, during which time oxygen physically dissolved in the blood supplies the animal's respiratory needs. The lowest levels occur during stages C<sub>1</sub> and C<sub>2</sub>, with A<sub>1</sub>, A<sub>2</sub>, B<sub>1</sub>, B<sub>2</sub>, and C<sub>3</sub> also characteristically possessing very little hemocyanin. Associated with the low concentrations of hemocyanin, Zuckerkindl found the oxygen partial pressure of the post-branchial blood of these stages to vary from 50 to 120 mm. Hg. In later stages he reported that the hemocyanin content rose and that the post-branchial oxygen pressures were lower (40–50 mm.). These latter pressures, as Zuckerkindl states, are somewhat uncertain and may be too high. This uncertainty resulted from calculations based on an oxygen equilibrium curve published by Hogben (1926) which may not represent physiological conditions. The total quantity of oxygen found in the post-branchial blood of *Maia* (0.10–0.52 vol.%) is about that reported for *Loxorhynchus* but is lower than that found in *Panulirus* and *Cardisoma*. It may or may not be significant that *Maia* and *Loxorhynchus* are both spider crabs and are rather sluggish as compared with the other two species.

Unlike Zuckerkindl, the author does not believe that the much higher post-branchial oxygen tensions reported for *Maia* indicate an important physiological difference for this species from those in which the post-branchial oxygen tensions are low. The high oxygen partial pressures in *Maia* blood are a direct consequence of the absence or low concentration of hemocyanin and do not necessarily indicate the oxygen tensions at which the tissues must operate. In the absence of a respiratory pigment, if the blood is to carry any appreciable quantity of oxygen away from the gills, it must carry it at a high partial pressure. Measurements of pre-branchial oxygen partial pressures in *Maia* would be of interest. It is difficult to assess the relative importance in blood of high oxygen tensions versus larger quantities of oxygen at lower pressures but it would seem that, so long as the tissues are capable of normal respiration at low pressures, the quantity rather than the tension would be of more importance. The intimate relationship between the blood and tissues would minimize any difficulties in the transfer of oxygen that might be expected to result from a small diffusion gradient between tissues and blood.

Why the hemocyanin should tend to disappear immediately following molt is not known. Zuckerkindl suggests that it may serve as a protein store. The period of most active tissue growth, stages C<sub>1</sub> and C<sub>2</sub>, coincides with the period during which the hemocyanin is least concentrated. Possibly this protein serves as a source of amino acids for the rapid growth that occurs at this time. That the hemocyanin can serve as a food source seems likely since this substance considerably



decreases in amount during starvation (unpublished observations on *Uca pugilator* and *Cardisoma guanhumi*). It would appear that during the period of presumably greatest gill permeability, when the hemocyanin would be least necessary as a respiratory pigment, this substance is used for another purpose. The primary question to be answered, however, is whether the findings for *Maia* are typical of the decapod Crustacea. In particular, it would be of interest to follow the hemocyanin level of the blood during the molt cycle of a more active species or one in which the hemocyanin concentration is characteristically high.

The hemocyanin in the blood of the decapod Crustacea may serve several functions. As a circulating protein, it is an important buffer and is largely responsible for the colloid osmotic pressure of the blood. Zuckerkandl's findings suggest that there may be other unknown functions. The very special properties of this substance, together with its location in the blood, however, make it difficult not to believe that its principal role is that of a respiratory pigment, whether it acts primarily as an oxygen transporter, to increase the oxygen capacity of the blood, or to speed the diffusion of oxygen. If this is not so, it seems highly improbable that its peculiar ability to combine reversibly with oxygen in appropriate pressure relationships would have evolved, or, having once developed, been perpetuated in such a large group of animals. This is not to say that it is necessarily essential for respiration. Probably all organisms may suffer the loss of various structures and continue to live although these structures normally play a role in the life of the organism and confer upon it certain advantages. The respiratory advantage conferred by hemocyanin consists of a greater supply of oxygen delivered to the tissues for a given quantity of circulatory work than would be delivered in its absence. Some of this extra oxygen may be used regularly; *Cardisoma* extracts more oxygen from a given volume of blood than could be carried in solution; or it may serve as a reserve which can be drawn upon during periods of greater activity or other stress.

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#### SUMMARY

1. The hemocyanin of the land crab, *Cardisoma guanhumi*, under physiological conditions, possesses a high oxygen affinity.
2. The oxygen equilibrium curve is sigmoid, with a fairly high degree of interaction occurring between the oxygen-combining sites of the hemocyanin molecule.
3. As in all other crustacean hemocyanins investigated, a normal Bohr effect is present.
4. Van Slyke analyses of the oxygen content of pre-brachial and post-brachial blood samples indicated that nearly all of the oxygen present was in combination

with the hemocyanin; consequently the oxygen partial pressures throughout the circulatory system were very low. The hemocyanin did not become saturated with oxygen during passage through the gills. These results are similar to those previously found for three other species of decapod crustaceans.

5. The carbon dioxide content of the blood was very high, averaging almost 50 volumes per cent.

6. It is shown that the low half-saturation value of the hemocyanin confers a stability of loading pressure in the face of changing environmental temperature. It is speculated that the high oxygen affinity may also represent an adaptation tending to counter the effect of water-conserving measures on the rate at which oxygen diffuses through the respiratory surfaces into the blood.

7. Zuckerkandl's studies on the crab, *Maia squinado*, are discussed and it is tentatively concluded that, though the hemocyanin of the decapod Crustacea may serve several functions and may not always be essential for respiration, its primary function is that of a blood respiratory pigment.

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